

DESCRIPTION

METHOD FOR TYPING OF HLA CLASS I ALLELES

5 Technical Field

HLA (Human Leukocyte Antigen) that is Human major histocompatibility antigen, is expressed on membranes of immunocompetent cells, presents processed peptides derived from exogenous and endogenous antigens to T lymphocytes, and functions as a marker to recognize self and non-self. The 10 present invention relates to a method, a reagent and a kit for typing of the HLA class I alleles. This invention is especially useful for judgement of compatibility between a donor and a recipient in organ transplantation, and for association analysis between the HLA class I genes and various types of diseases in the clinical and medical field. This invention enables us to 15 easily automate and mechanize detection and determination of the HLA class I alleles.

Background of Art

Typing of the HLA antigens has been mainly performed by the serological 20 method using human alloantibodies. By using the specific antibodies to each HLA antigen which are contained in cord blood or serum from subjects who have frequently undergone blood transfusion, complement-mediated cytotoxicity is caused in the antigen-antibody reaction. It changes permeability of positive cell membranes to take an eosin pigment into the cell, resulting 25 in being detected as colored and expanding cells with a microscope. It is possible to type HLA-A, HLA-B and HLA-C antigens belonging to HLA class I, and HLA-DR and HLA-DQ antigens belonging to HLA class II by this method.

However, this method has problems in terms of collection, quality control and supply of the specific antibodies. Furthermore, the survival rate of cells is utilized as an indicator for judgement in this method. Therefore, poor conditions of subjects, for example, a low survival rate of cells caused 5 by disease or influence by passage of time after blood collection, lead to decrease of credibility for results of testing.

In recent years, a development of molecular biotechnology has enabled us to analyze the region of genes encoding the HLA antigens. That has clarified the correspondence between the HLA antigens and the sequences of 10 the HLA genes. This means it has been possible to identify the HLA antigen type by analyzing the specific sequences of the HLA genes (DNA typing). Especially, PCR (polymerase chain reaction) method which can high-sensitively detect a slight change of sequences is utilized to type the HLA-DR, -DQ, or -DP genes belonging to HLA class II. Several PCR-based typing 15 methods for HLA class II DNA such as PCR-SSOP (Sequence-Specific Oligonucleotide Probe) method, PCR-RFLP (Restriction Fragment Length Polymorphism) method, PCR-SSP (Sequence-Specific Primers) method and PCR-SSCP (Single Strand Conformation Polymorphism) method have been developed. In all these methods, the gene region to analyze is amplified 20 by the PCR method and then the variable region in the sequences of the amplified products is analyzed by combination with another methods in order to distinguish the genotype. The HLA class II DNA typing method makes it possible to classify the HLA type at the allele level in addition to classification by the classical serological method using human alloantisera.

25 Development of the PCR based-method for HLA class I DNA typing is delayed remarkably, comparing with HLA class II typing. The reasons are as follows:
(1) While almost all the class II gene mutations (gene substitutions),

including those which reflect the specificity of antigens, concentrate in the region of the exon 2, the class I gene mutations are interspersed among the regions of the exons 2 and 3, or the exon 4. (2) The HLA class I genes, including non-classical genes (HLA-E, -F and -G) and pseudogenes (HLA-H, -J, -K and -L), are highly homologous among them.

To date, several HLA class I DNA typing methods have been reported. However, all these methods require complicated manipulation, strict reaction condition and skill. Those are not suitable for handling a large number of samples and offer only low resolution HLA typing. Furthermore, the typing methods for each gene are not standardized.

Disclosure of Invention

The purpose of this invention is to solve problems of the manipulation of HLA class I locus antigen typing by the classical serological method, and 15 to produce a method, a kit and a reagent for classifying the subtype of the HLA class I antigens at the allele level (allele typing), which has not been distinguished by the classical method. Furthermore, the aim of this invention is to provide a method for typing of the HLA class I alleles which can automate and mechanize easily.

As a result of intensive studies for these subjects, the inventors have established primers which can amplify all the HLA-A alleles, all the HLA-B alleles or all the HLA-C alleles and specific primers to the common sequences among all alleles in the group consisting of the specific HLA-A alleles or the specific HLA-B alleles. The inventors have established probes which can 25 specifically hybridize with the sequence of at least one specific HLA-A allele, at least one specific HLA-B allele or at least one specific HLA-C allele. The inventors have found out that it is possible to distinguish the HLA class

I antigen or allele, by hybridizing the PCR amplified products derived from the specific HLA class I allele or the specific group with the DNA probes described above which are immobilized on wells of microtiter plates, adding an enzyme-conjugate which can specifically bond to a label of the amplified 5 products at the same time as or after the hybridization, and adding a chromogenic substrate, a luminescent substrate or a fluorescent substrate to the mixture, to detect as signals whether or not the amplified products are hybridized with the immobilized DNA probes. Thus, they have accomplished this invention.

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The main embodiment of this invention is a method for typing of HLA class I alleles, which comprises the following steps from (a) to (d).

15 (a) A step, using HLA class I gene or nucleic acids containing their fragment for a template,

(1) To non-selectively amplify all HLA-A alleles, all HLA-B alleles or all HLA-C alleles by a PCR method using a primer pair which can amplify all the HLA-A alleles, all the HLA-B alleles or all the HLA-C alleles, or

20 (2) To selectively amplify a specific group consisting of specific HLA-A alleles or specific HLA-B alleles by a PCR method using a primer pair which is specific to the common sequence to alleles of the specific group consisting of the specific HLA-A alleles or the specific HLA-B alleles,

25 (b) A step to add the above products amplified by the PCR method to wells of microtiter plates, wherein each well is modified with a carboxyl group to covalently immobilize amino-modified DNA probes which can specifically hybridize with the sequence of at least one specific HLA-A allele, at least one specific HLA-B allele or at least one specific HLA-C allele, and to

hybridize the amplified products with the immobilized DNA probes, wherein the DNA probes are selected depending on the above amplified specific HLA class I gene or group;

(c) A step to detect as signals whether or not the amplified products are
5 hybridized with the immobilized probes; and

(d) A step to determine the type of the HLA class I allele based on the signal pattern detected at the step (c) according to the Typing Table.

The PCR amplification of the target gene at the step (a), can be
10 classified into 2 steps. One is a step to non-selectively amplify all the HLA-A alleles, all the HLA-B alleles or all the HLA-C alleles by the PCR method using a primer pair which can amplify all the HLA-A alleles, all the HLA-B alleles or all the HLA-C alleles. The other is a step to selectively amplify the specific group consisting of the specific HLA-A allele group or the
15 specific HLA-B allele group by the PCR method using a primer pair which is specific to the common sequences to alleles of the specific group consisting of the specific HLA-A alleles or the specific HLA-B alleles. At the former step, PCR primers are designed to be specific to the common sequences, which are within the region of all alleles belonging to the HLA-A allele, the HLA-B
20 allele or the HLA-C allele, or ahead and behind the region. At the latter step, PCR primers are designed to be specific to the common sequences to all alleles included in the specific group in order to amplify the specific group. When the specific group is selectively amplified in the presence of some groups, the primers described above don't need to be used for both a sense
25 primer and an antisense primer of a primer pair corresponding to the specific group. It is possible to use the specific primer to the specific group for one of primers and the specific primer to all the groups for the other. The

latter step can be performed according to the reference described by the inventors (Tissue Antigens 1997, Vol.50, 535-545). A method to selectively amplify alleles encoding the HLA-A2 antigen or the HLA-B40 antigen as a group is disclosed in the present description.

5 At the step (a), the PCR-amplified products derived from the allele belonging to the HLA-A alleles, the HLA-B alleles or the HLA-C alleles, or from the specific group, are produced. But it is not possible to distinguish the type of the HLA class I allele at the step. The hybridization reaction at the step (b) using the specific DNA probes is applied to the following
10 steps.

The Typing Table at the step (d) is made using signal patterns obtained by hybridizing the PCR amplified products from samples whose HLA class I antigen types or allele types are known, with DNA probes which can specifically hybridize with the sequence of at least one specific HLA class
15 I allele. Persons skilled in the art can make easily the Typing Table. As the Typing Table, Figures 1 to 6 can be referred. If someone wants to use DNA probes, which are not described in this description, another Typing Table can be used. The Typing Table is made from signal patterns obtained by hybridizing the PCR amplified products from samples whose HLA class I antigen
20 types or allele types are known, with another DNA probe. As described above, persons skilled in the art can also make easily these Typing Tables. It should be considered that each sample has the HLA class I allele type in a homozygous or heterozygous state, when the HLA class I allele type is distinguished according to the Typing Tables.

25 In a preferable embodiment, the PCR method at the step (a) is performed by using a primer pair in which at least one of them is labeled, in order to detect whether or not the amplified products hybridize with immobilized

DNA probes as signals at the step (c) described above. In the other embodiment, the above PCR can be performed by using 4 kinds of deoxyribonucleotide triphosphate (dNTP) in which at least one of them is labeled. As a substance used for labeling, a radioisotopic substance, or 5 a non-radioisotopic substance such as a biotin or a digoxigenin, can be utilized.

In a preferable embodiment, at the step (b) or (c) described above, the hybridization of the products amplified by the PCR method with the immobilized DNA probe is performed by adding an enzyme-conjugate which can specifically bond to a label of the amplified products is added at the same time as hybridization or after, and the amplified products hybridizing with the immobilized DNA probe is detected as signals by adding a chromogenic substrate, a luminescent substrate or a fluorescent substrate which can specifically react with the enzyme. When a peroxidase-conjugated streptavidin is used 10 as an enzyme-conjugate, the signal can be immediately detected after washing 15 by adding an enzyme-conjugate at the same time as hybridization.

In a preferable embodiment, at least one of a primer pair at the step (a) described above is biotinylated, and an enzyme-conjugate which can specifically bond to the biotinylated label at the step (b) or (c) is an 20 enzyme-conjugated streptavidin, for example, a peroxidase-conjugated streptavidin or an alkaline phosphatase-conjugated streptavidin.

In a preferable embodiment, the hybridization of the products amplified by the PCR method with immobilized DNA probes is performed in a solution containing formamide at the step (b) described above. The formamide 25 concentration of the solution described above (hybridization buffer) is from 5% to 30%, and from 10% to 25% as a preferable concentration. The concentration can be changed according to the sequence, the length and the

type of the used DNA probe. The most preferable formamide concentration is about 20%.

In a preferable embodiment, the hybridization at the step (b) is performed in a solution containing formamide at the temperature of the 37°C. 5 The preferable temperature is from 32°C to 42°C. The temperature can be changed according to the sequence, the length and the type of the used DNA probe as mentioned above for the formamide concentration. The most desirable temperature is about 37°C. Hybridization is usually performed at comparatively high temperature, at about 65°C, to improve the specificity. 10 By using the solution containing formamide, the reaction can be performed at low temperature, at about 37°C.

In a preferable embodiment, when the solution containing formamide is used for the hybridization at the step (b) described above, the temperature for washing after hybridization of the amplified products by the PCR method 15 with immobilized DNA probes and/or after binding a label of the amplified products with an enzyme-conjugate is performed at room temperature. Namely, washing can be performed at low temperature like room temperature as by using the solution containing formamide, as well as the above hybridization.

The amino-modified DNA which can specifically hybridize with at least 20 one specific HLA-A allele, used at the step (b) in this invention, can be selected from the group consisting of A98T (SEQ ID No.:1), A98A (SEQ ID No.:2), A160A (SEQ ID No.:3), A239A (SEQ ID No.:4), A238A (SEQ ID No.:5), A240T (SEQ ID No.:6), A257TC (SEQ ID No.:7), A259AC (SEQ ID No.:8), A270T (SEQ ID No.:9), A282C (SEQ ID No.:10), A290T (SEQ ID No.:11), A299T (SEQ ID No.:12), A302G 25 (SEQ ID No.:13), A355G (SEQ ID No.:14), A362TA (SEQ ID No.:15), A362TT (SEQ ID No.:16), A368A (SEQ ID No.:17), A368G (SEQ ID No.:18), A368T (SEQ ID No.:19), A402G (SEQ ID No.:20), A423T (SEQ ID No.:21), A448C (SEQ ID No.:22), A485A

(SEQ ID No.:23), A524G (SEQ ID No.:24), A526T (SEQ ID No.:25), A527A (SEQ ID No.:26), A538CG (SEQ ID No.:27), A539A (SEQ ID No.:28), A539T (SEQ ID No.:29), A555T (SEQ ID No.:30), A559G (SEQ ID No.:31), A570CG (SEQ ID No.:32), A570GT (SEQ ID No.:33), A779A (SEQ ID No.:34), A843A (SEQ ID No.:35), A34 5 (SEQ ID No.:100), A282CT (SEQ ID No.:101), A290TR (SEQ ID No.:102), A302GR (SEQ ID No.:103), A414A (SEQ ID No.:104), A468T (SEQ ID No.:105), A489A (SEQ ID No.:106), A502C (SEQ ID No.:107), A538TG (SEQ ID No.:108) and complementary strands thereof.

The amino-modified DNA probe which can specifically hybridize with at 10 least one specific HLA-B allele can be selected from the group consisting of BL1 (SEQ ID No.:36), BL3 (SEQ ID No.:37), BL4 (SEQ ID No.:38), BL5 (SEQ ID No.:39), BL9 (SEQ ID No.:40), BL10 (SEQ ID No.:41), BL11 (SEQ ID No.:42), BL24 (SEQ ID No.:43), BL25 (SEQ ID No.:44), BL34 (SEQ ID No.:45), BL35 (SEQ ID No.:46), BL36 (SEQ ID No.:47), BL37 (SEQ ID No.:48), BL38 (SEQ ID No.:49), 15 BL39 (SEQ ID No.:50), BL40 (SEQ ID No.:51), BL41 (SEQ ID No.:52), BL42 (SEQ ID No.:53), BL56 (SEQ ID No.:54), BL57 (SEQ ID No.:55), BL78 (SEQ ID No.:56), BL79 (SEQ ID No.:57), BL222A (SEQ ID No.:58), BL272GA (SEQ ID No.:59), BL226G (SEQ ID No.:60), BL292G (SEQ ID No.:61), BL292T (SEQ ID No.:62), BL361G (SEQ ID No.:63), BL409T (SEQ ID No.:64), BL512T (SEQ ID No.:65), BL538CG (SEQ ID 20 No.:66), BL538G (SEQ ID No.:67), BL39R (SEQ ID No.:109), BL50 (SEQ ID No.:110), BL77 (SEQ ID No.:111), BL272A (SEQ ID No.:112), BL263T (SEQ ID No.:113), BL527A (SEQ ID No.:114), BL570GT (SEQ ID No.:115) and complementary strands thereof.

The amino-modified DNA probe which can specifically hybridize with at 25 least one specific HLA-C allele can be selected from the group consisting of CC (SEQ ID No.:68), A-12 (SEQ ID No.:69), A-2 (SEQ ID No.:70), A-3 (SEQ ID No.:71), A-4 (SEQ ID No.:72), A-54 (SEQ ID No.:73), B-1 (SEQ ID No.:74),

B-2 (SEQ ID No.:75), C-12 (SEQ ID No.:76), C-24 (SEQ ID No.:77), C-33 (SEQ ID No.:78), C-43 (SEQ ID No.:79), 134-g (SEQ ID No.:80), 134-A2 (SEQ ID No.:81), 353TCA1 (SEQ ID No.:82), 343A (SEQ ID No.:83), RA-2 (SEQ ID No.:116), RA-41 (SEQ ID No.:117), RB-28 (SEQ ID No.:118), 201g1 (SEQ ID No.:119), C206gR (SEQ ID No.:120), R341A (SEQ ID No.:121), R343g3 (SEQ ID No.:122), 353TCC (SEQ ID No.:123), 361T1 (SEQ ID No.:124), 361T368g (SEQ ID No.:125), 361T368T1 (SEQ ID No.:126), 369C (SEQ ID No.:127), 387g1 (SEQ ID No.:128), 526AC2 (SEQ ID No.:129), 538gAC (SEQ ID No.:130) and complementary strands thereof.

This invention also comprises the DNA probe itself (from SEQ ID No.: 1 to SEQ ID No.:83 and from SEQ ID No.:100 to SEQ ID No.:130) which can specifically hybridize with at least one specific HLA-A allele, at least one specific HLA-B allele or at least one specific HLA-C allele for using the method for distinguishing the HLA class I allele type.

Both an amino-modified DNA probe and an unmodified DNA probe can be used. However, when the probe is covalently immobilized on wells of carboxylate-modified microtiter plates, the amino-modified probe must be used. Some bases can be deleted from or added to the end of the DNA probe within the range that the DNA probe can specifically hybridize with at least one specific HLA-A allele, at least one specific HLA-B allele or at least one specific HLA-C allele, namely, within the range that the DNA probe can keep the original specificity of hybridization. Accordingly, the DNA probes in this invention also comprise DNA probes wherein some bases are deleted from or added to the nucleic acid sequence from SEQ ID No.:1 to SEQ ID No.:83 and SEQ ID No.:100 to SEQ ID No.:130 within the range described above.

The primers which can amplify all the HLA-A alleles, all the HLA-B alleles or all the HLA-C alleles at the step (a) in this invention, can be selected from the group consisting of CGA011 (SEQ ID No.:90), CGA012 (SEQ

10 ID No.:91), AIn3-66C (SEQ ID No.:92), 5BCIn37-34C (SEQ ID No.:96), 5BCIn37-24g (SEQ ID No.:97) and 5BCIn37-34g2 (SEQ ID No.:99). The primer which is specific to the common sequence to alleles of the specific group consisting of the specific HLA-A alleles or the specific HLA-B alleles, can 5 be selected from A2-5T (SEQ ID No.:84), A3-273T (SEQ ID No.:85), A4-8C (SEQ ID No.:86), A4-254G (SEQ ID No.:87), BASF-1 (SEQ ID No.:88), and BASR-1 (SEQ ID No.:89). This invention comprises the primer itself described above (from SEQ ID No.:88 to SEQ ID No.:92, from SEQ ID No.:96 to SEQ ID No.:97 and SEQ ID No.:99), used for the method to type the HLA class I alleles.

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Novel HLA-A alleles, HLA-B alleles and HLA-C alleles have been discovered. In the report of the WHO (World Health Organization) Nomenclature Committee for the HLA system, 82, 186, and 42 of alleles have been assigned for the HLA-A, -B and -C loci, respectively, at March 1997. 15 This invention can discriminate all these alleles. Furthermore, the method shown in this invention, together with an optional, easy-performed improvement, such as adding extra DNA probes or primers, can cope with discrimination of alleles which may be discovered and enrolled in the future.

20 This invention can provide a kit and a reagent for typing of the HLA class I alleles described in this description. Furthermore, this invention can provide a kit and a reagent which comprise the DNA probes and the primers described in this description. For example, the kit can comprises a solution containing the primers (from SEQ ID No.:84 to SEQ ID No.:92, from SEQ ID No.:96 to SEQ ID No.:97 and SEQ ID No.:99) which is disclosed in this invention, PCR buffer solution, which may be concentrated solution, dNTPs, thermostable DNA polymerase, the DNA probes (from SEQ ID No.:84 to SEQ ID No.:92, from

SEQ ID No.:96 to SEQ ID No.:97 and SEQ ID No.:99) which is disclosed in this invention or a microtiter plate on whose wells the DNA probes are covalently immobilized, a denature solution, a hybridization buffer, a washing solution and an instruction for the kit which includes the Typing Tables. The primer 5 described above can optionally be labeled with a radioisotopic or non-radioisotopic substance. The primers can form a primer pair. The solution containing the primer can be freeze-dried. When the primer is not labeled, at least one of four kinds of dNTPs must be labeled. When a non-radioisotopic substance is used as a label, an enzyme-conjugate solution, a chromogenic 10 reagent including a chromogenic substrate and a chromogenic solution, a luminescent reagent or a fluorescent reagent, a stop solution and so on can be added as a component in the kit. Furthermore, a component such as guanidine thiocyanate buffer for isolation of genome DNAs, can be optionally added in the kit to the degree promoting enforcement of this invention.

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Brief Description of Figures

Figure 1 indicates a Typing Table showing the reaction pattern between samples which the HLA-A2 allele type is known and DNA probes in the present invention. Each name of DNA probes is shown on the top in the Figure, and 20 each type of the HLA-A2 alleles is shown on the left side in the Figure. Closed square and Open square mean a positive and a negative reaction, respectively.

Figure 2 indicates a Typing Table showing the reaction pattern between samples which the HLA-B40 allele type is known and DNA probes in the present 25 invention. Each name of DNA probes is shown on the top in the Figure, and each type of the HLA-B40 alleles is shown on the left side in the Figure. Closed square and Open square mean a positive and a negative reaction,

respectively.

Figure 3 indicates a Typing Table showing the reaction pattern between samples which the HLA-A antigen and allele type are known, and DNA probes in the present invention. Each name of DNA probes is shown on the top in the Figure, and each type of the HLA-A antigens and alleles is shown on the left side in the Figure. Closed square and Open square mean a positive and a negative reaction, respectively.

Figure 4 and 5 indicate Typing Tables showing the reaction pattern between samples which the HLA-B antigen and allele type is known, and DNA probes in the present invention. Each name of DNA probes is shown on the top in the Figures, and each type of the HLA-B antigens and alleles is shown on the left side in the Figures. Closed square and Open square mean a positive and a negative reaction, respectively.

Figure 6 indicates a Typing Table showing the reaction pattern between samples which the HLA-C antigen and/or allele type is known, and DNA probes in the present invention. Each name of DNA probes is shown on the top in Figure, and each type of the HLA-C and/or alleles is shown on the left side in Figure. Closed square and Open square mean a positive and a negative reaction, respectively.

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The Best Mode for Carrying Out the Invention

The strategy of this invention described above is explained in more detail.

25 The typing method in this invention can be explained, dividing into the following 6 steps.

- 1)Extraction of chromosome(genome) DNAs,
- 2)PCR amplification of target genes,

- 3)Immobilization of DNA probes on wells of microtiter plates,
- 4)Hybridization of PCR products with DNA probes,
- 5)Detection of signals, and
- 6)Determination of the allele type.

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1)Extraction of chromosome(genome) DNAs

A method for preparation of genome DNAs is explained as follows. Leukocytes are isolated from collected blood according to usual methods and are lysed in a guanidine thiocyanate buffer. Proteins are eliminated by 10 phenol extraction. A sodium acetate buffer (pH 5.2) is added and mixed. Genome DNAs are obtained by adding chilled ethanol.

2)PCR amplification of target genes

The region containing the HLA class I allele is amplified by the PCR 15 method using genome DNAs described above for a template. Commercialized reagents can be used for amplification described above. Amplification can be performed according to attached instructions. If it is necessary, reaction temperature, reaction time, the number of cycles and so on can be changed. Then, the amplification is performed by using a primer pair for 20 a reaction tube. Amplification by adding multiple primer pairs into the same reaction tubes, can decrease operation task or cost. From the viewpoint of the purpose of this invention, a primer pair which one of them is biotinylated, is used for the practical testing or a kit.

For example, A2-5T and 5'-biotinylated A3-273T can be used for a primer 25 pair to amplify the region containing the exon 2, the intron 2 and the exon 3 of the HLA-A2 alleles by the PCR method. A4-8C and 5'-biotinylated A4-254G can be used for a primer pair to amplify the region containing the exon 4

of the HLA-A alleles by the PCR method. These primers are described in the reference of the inventors (Tissue Antigens 1997, described above) .

For example, BASF-1 and 5'-bitinylated BASR-1 can be used for a primer pair to amplify the region containing the exon 2, the intron 2 and the exon 5 3 of the HLA-B40 alleles by the PCR method.

For example, CGA011 or CGA012, and 5'-biotinylated AIn3-66C can be used for a primer pair to amplify the region containing the exon 2, the intron 2 and the exon 2 3 of all the HLA-A alleles by the PCR method.

For example, 5BIN1-TA (SEQ ID No.:93) or 5BIN1-CG (SEQ ID No.:94) , 10 and 5'-biotinylated 3BIN3-37 (SEQ ID No.:95) can be used for a primer pair to amplify the region containing the exon 2, the intron 2 and the exon 3 of all the HLA-B alleles by the PCR method. The primers are described in the reference of Cereb N. et al (Tissue Antigens 1997, Vol.50, 74-76) .

For example, 5BCIn37-34C, 5BCIn37-24g or 5BCIn37-34g2, and 5'- 15 biotinylated 3BCIn3-12 (SEQ ID No.:98) can be used for a primer pair to amplify the region containing the exon 2, the intron 2 and the exon 3 of all the HLA-C alleles by the PCR method. The primer, 3BCIn3-12, is described in the reference of Cereb N. et al (Tissue Antigens 1995, Vol.45, 1-11) .

20 3)Immobilization of DNA probes on wells of microtiter plates

Amino-modified DNA probes (1-20 pmol) which can specifically hybridize with the sequence of at least one specific HLA-A allele, at least one specific HLA-B allele or at least one specific HLA-C allele, are added onto each well 25 of carboxylate-modified polystyrene microtiter plates and immobilized covalently by inducing the chemical amino-binding reaction using a suitable catalyst, for example, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC). After immobilization of the DNA probes on wells, microtiter plates are washed

with a suitable buffer. After washing, microtiter plates can be stored over an extended period of time on wet and cold condition.

4) Hybridization of PCR products with DNA probes

5 The PCR amplified products are denatured to a single strand DNA under strong alkali, for example, NaOH, and are hybridized with DNA probes which are immobilized on wells of microtiter plates. The hybridization is performed in a solution containing about 20 % formamide on hybridization condition at about 37°C. After the hybridization, excessive amplified
10 products or those which don't have the specific sequence to DNA probes described above, are eliminated. DNA probes used at this step are selected in compliance with the specific HLA class I gene or the specific group which are amplified at the above step.

For example, as for the amplified products from the region containing
15 the exon 2, the intron 2 and the exon 3 of the HLA-A2 alleles by a primer pair described above, A2-5T and A3-273T, or the amplified products from the exon 4 of the HLA-A alleles by a primer pair, A4-8C and A4-254G, the hybridization can be performed by using A98T, A98A, A160A, A240T, A270T, A290T, A355G, A362TA, A362TT, A368A, A368G, A368T, A402G, A485A, A527A, A539A, A539T,
20 A559G, A570CG, A779A or A843A for DNA probes.

For example, as for the amplified products from the region containing the exon 2, the intron 2 and the exon 3 of the HLA-B40 alleles by a primer pair described above, BASF-1 and BASR-1, the hybridization can be performed by using BL4, BL5, BL24, BL25, BL34, BL35, BL37, BL39, BL41, BL50, BL56, BL57,
25 BL222A, BL409T or BL512 for DNA probes.

For example, as for the amplified products from the region containing the exon 2, the intron 2 and the exon 3 of all the HLA-A alleles by a primer

pair described above, CGA011, CGA012 or AIn3-66C, the hybridization can be performed by using A34, A239A, A238A, A257TC, A259AC, A282C, A282CT, A290TR, A299T, A355G, A414A, A448C, A468T, A489A, A502C, A526T, A538CG, A538TG, A539A, A539T, A555T, A570CG, A570GT or A302GR for DNA probes.

5 For example, as for the amplified products from the region containing the exon 2, the intron 2 and the exon 3 of all the HLA-B alleles by a primer pair described above, 5BIN1-TA, 5BIN1-CG or 3BIN3-37, the hybridization can be performed by using BL1, BL3, BL4, BL9, BL10, BL11, BL34, BL36, BL37, BL38, BL39R, BL40, BL41, BL42, BL77, BL78, BL79, BL226G, BL263T, BL272A, BL527A, 10 BL538CG, BL538G or BL570GT for DNA probes.

For example, as for the amplified products from the region containing the exon 2, the intron 2 and the exon 3 of all the HLA-C alleles by a primer pair described above, 5BCIn37-34C, 5BCIn-37-24g, 5BCIn37-34g2 or 5BCIn3-12, the hybridization can be performed by using 201g1, C206gR, A-12, RA-15 2, A-3, RA-41, A-54, B-1, RB-28, C-12, C-24, C-33, C-43, 134-g, 134-A2, 353TCA1, 343A, R341A, R343g3, 353TCC, 361T1, 361T368g, 361T368T1, 369C, 387g1, 526AC2 or 538gAC for DNA probes.

About the concrete type of the HLA class I allele which are distinguished by the hybridization with these DNA probes, examples and Figures can be 20 referred.

Besides these DNA probes, A302G, A423T, A524G, BL272GA, BL292G, BL292T, BL361G, CC, A-2, A-4 or B-2 can be used for typing of the HLA class I antigens or alleles described below. A302G, A423T and A524G can specifically hybridize with the sequence of the HLA-A antigens or alleles, A*2501 and 25 A*3201, A*2501, A26, A34, A*4301 and A66, and A*2301, A29, A*31012, A*3201, A33 and A*7401, respectively. BL272GA, BL292G, BL292T and BL361G can specifically hybridize with the sequence of the HLA-B antigens or alleles,

B14, B38 and B39, B7, B8, B14, B27, B39, B*4201, B*4601, B*5401, B55, B56, B67, B*7301, B*7801 and B*8101, B13, B15, B18, B35, B37, B38, B40, B41, B44, B*4501, B*4701, B48, B*4901, B*5001, B51, B52, B*5301, B57, B58, B*5901 and B*7802, and B57, respectively. CC can hybridize with the sequence of all 5 the HLA-C alleles. A-2, A-4 and B-2 can specifically hybridize with the sequence of the HLA-C antigens or alleles, Cw2, Cw3, Cw*0403 and Cw15, Cw*0602, Cw7 and Cw18, and Cw1, Cw3, Cw7, Cw8, Cw*1202, Cw*1203, Cw*1301, Cw*14, Cw*1601 and Cw*16041, respectively.

10 5)Detection of signals

An example for detection of signals is explained below. The PCR amplified products hybridizing with DNA probes can be detected by utilizing a label, which they have in themselves, such as a biotin. After an alkaline phosphate-conjugated streptavidin or a peroxidase-conjugated streptavidin 15 which can bond to a biotin, is added to each well of the microtiter plates, and the plates are sealed, the reaction is performed by incubation on proper temperature condition. The hybridizing amplified products are detected as signals by using a chromogenic substrate such as *p*-nitrophenylphosphate (PNPP) or 3,3',5,5'-tetramethylbenzidine (TMB). Detection of signals is 20 performed by measurement of the absorbance. The signals described above can be automatically detected by using a machine, and those by color development can be easily detected by the naked eye.

6)Determination of the allele types

25 By signal patterns which are detected on the microtiter plate described above, for example, in compliance with the Typing Tables which are disclosed in Figures 1-6, the HLA class I alleles are determined. Patterns of these

Typing Tables in Figures 1-6 can be arranged in case of necessity.

Examples

This invention is explained in more detail by showing examples, which
5 are actually performed by using samples, whose HLA types are known. However,
the range of this invention is not limited to only these examples.

Example 1 HLA-A2 allele typing

Leukocytes (Samples 1-4) which were isolated from peripheral blood
10 (about 10ml) of normal subjects according to usual methods, were lysed in
500 μ l of guanidine thiocyanate buffer (4M guanidine thiocyanate, 25mM sodium
citrate(pH7.0), 0.5% sodium N-lauroylsarcosinate, 1% mercaptoethanol).
The solution was extracted twice with phenol to eliminate proteins. After
mixing with 3M sodium acetate buffer (pH 5.2), genome DNAs were obtained by
15 adding twice volume of chilled ethanol. By using this DNAs, typing of the
HLA-A2 alleles was performed as follows.

By using A2-5T and 5'-biotinylated A3-273T for a primer pair,
amplification of the region containing the exon 2, the intron 2 and the exon
3 of the HLA-A2 alleles from DNAs described above was performed by the PCR
20 method. Likewise, by using A4-8C and 5'-biotinylated A4-254G for a primer
pair, amplification of the region containing the exon 4 of the HLA-A alleles
was also performed by the PCR method. The reaction solution was composed
of genomic DNAs (100 ng), 1.4 units of thermostable DNA polymerase which was
pretreated with Taq StartTMAntibody for 5 min at room temperature, 67mM
25 Tris-HCl (pH 8.8), 16.6 mM ammonium sulfate, 1.5 mM magnesium chloride, 0.01%
Tween 20, 200 μ M dNTPs, and each 1.7 μ M of a primer pair in a final volume
of 80 μ l. DNA amplification was performed by using GeneAmp PCR system 9600

(Perkin Elmer) by initial denaturation at 95°C for 2 min followed by 5 cycles of denaturation for 25 s, annealing at 70°C for 45 s, extension at 72°C for 45 s followed by 36 cycles of denaturation for 25 s, annealing at 65°C for 50 s, extension at 72°C for 45 s.

5 5'-amino-modified DNA probes, A98T, A98A, A160A, A240T, A270T, A290T, A355G, A362TA, A362TT, A368A, A368G, A368T, A402G, A485A, A527A, A539A, A539T, A559G, A570CG, A779A and A843A, were immobilized covalently on wells of carboxylate-modified polystyrene microtiter plates as follows. Twenty-five μ l of the DNA probes described above which were dissolved with sterile 10 distilled water, was added to each of 20 wells which were used for a sample, in order shown in Figure 1. Next, 75 μ l of 0.2M 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) was added to each well and mixed. After the plates were sealed and incubated for 16 hours at room temperature, they were washed four times with PBS buffer (7.5mM di-potassium 15 hydrogenphosphate, 2.5mM potassium dihydrogenphosphate, 0.15M sodium chloride). Two hundreds μ l of 0.4N NaOH were added to each well and the plates were incubated for 1 hour at 37°C. The plates were washed four times with PBS buffer.

One hundred μ l of GMC buffer for hybridization (0.25M di-sodium 20 hydrogenphosphate, 7% SDS, 1% BSA, 0.5M EDTA, 0.03M phosphoric acid, 20% formamide) was added to each well of the microtiter plates and the plates were incubated for 5 min at 37°C. After incubation, the buffer was removed from each well. During incubation, 72 μ l of the amplified products which 25 were obtained from the region containing the exon 2, the intron 2 and the exon 3, and 8 μ l of the amplified products which were obtained from the region containing the exon 4, were denatured with an equivalent volume of 0.4 NaOH for 5 min at room temperature. After denaturation, 1800 μ l and 200 μ l of

hybridization buffer were added to the denatured products, respectively, mixed and 100 μ l of them was added to each well (the former was added Well 1 to Well 18. The latter was added to Well 19 and Well 20). The microtiter plates were sealed and incubated for 1 hour at 37°C.

5 The microtiter plates were sealed and incubated for 45 min at 37°C. After the solution was removed from wells, the plates were washed five times with 2 \times SSC washing solution(0.3M sodium chloride, 0.03M tri-sodium citrate), 100 μ l of alkaline phosphatase-conjugated streptavidin (Gibco BRL) solution, diluted to 1/1000 in TTBS enzyme diluting solution (0.2M Tris-HCl(pH7.6), 10 0.5M sodium chloride, 0.5% Tween 20), was added to each well. After the solution was removed from wells, the plates were washed five times with the washing solution described above, chromogenic substrate solution (4mg/ml PNPP (*p*-nitrophenylphosphate), 1mM magnesium chloride, 10% diethanolamine (pH9.8)) was added and incubated for 30 min at 37°C. After incubation, color 15 development was stopped by adding 25 μ l of 0.5M EDTA to each well and the absorbance was measured at 405 nm. The absorbance to each sequence is shown in Table 1. The absorbance of positive and negative signals was 1.0 and over, and under 0.5, respectively. By using these results, HLA-A2 allele typing for each sample (1 – 4) was performed according to the Typing Table shown 20 in Figure 1. The typing results are shown in the bottom column of Table 1 as follows.

Table 1

Results of HLA-A2 allele typing (the absorbance at 405nm)

Well	SS0 probe	Sample 1	Sample 2	Sample 3	Sample 4
1	A240T	1.894	1.907	2.049	1.849
2	A368A	1.675	1.744	0.116	1.210
3	A368G	0.265	0.294	2.050	0.198
4	A368T	0.077	0.212	0.038	0.065
5	A362TT+A362TA	0.282	0.261	0.052	0.202
6	A98T	1.655	0.084	1.768	1.406
7	A98A	0.047	1.871	0.038	1.589
8	A539T	1.952	1.971	1.974	1.127
9	A539A	0.267	0.280	0.380	0.232
1 0	A402G	0.299	0.344	0.326	0.227
1 1	A527A	0.199	0.212	0.229	0.140
1 2	A270T	0.194	0.265	0.263	0.229
1 3	A290T	0.118	0.104	0.105	0.112
1 4	A559G	0.027	0.019	0.026	0.048
1 5	A485A	0.171	0.176	0.169	0.108
1 6	A355G	1.956	1.971	1.877	1.344
1 7	A160A	0.024	0.024	0.030	0.030
1 8	A570CG	0.040	0.027	0.050	0.064
1 9	A779A	0.020	0.021	0.034	0.041
2 0	A843A	0.025	0.049	0.038	0.045
HLA-A2 Allele type		A*0201	A*0206	A*0207	A*0201/ 0206

Example 2 HLA-B40 allele typing

Leukocytes (Samples 5-8) which were isolated from peripheral blood (about 10ml) of normal subjects according to usual methods, were lysed in 5 500 μ l of guanidine thiocyanate buffer (4M guanidine thiocyanate, 25mM sodium citrate(pH7.0), 0.5% sodium N-lauroylsarcosinate, 1% mercaptoethanol). The solution was extracted twice with phenol to eliminate proteins. After mixing with 3M sodium acetate buffer (pH 5.2, genome DNAs were obtained by adding twice volume of chilled ethanol. By using this DNAs, typing of the 10 HLA-B40 alleles was performed as follows.

By using BASF-1 and 5'-biotinylated BASR-1 for a primer pair, amplification of the region containing the exon 2, the intron 2 and the exon 3 of the HLA-B40 alleles from DNAs described above was performed by the PCR method. The reaction solution was composed of genomic DNAs (100 ng), 1.4 15 units of thermostable DNA which was pretreated with Taq StartTMAntibody for 5 min at room temperature, 33.5mM Tris-HCl (pH 8.8), 8.8 mM ammonium sulfate, 1.5 mM magnesium chloride, 0.005% Tween 20, 200 μ M dNTPs, and each 1.7 μ M of a primer pair in a final volume of 70 μ l. DNA amplification was performed by using GeneAmp PCR system 9600 (Perkin Elmer) by initial denaturation at 20 95°C for 2 min followed by 5 cycles of denaturation for 25 s, annealing at 70 °C for 45 s, extension at 72°C for 45 s followed by 36 cycles of denaturation for 25 s, annealing at 65°C for 50 s, extension at 72°C for 45 s.

25 5'-amino-modified DNA probes, BL4, BL5, BL24, BL25, BL34, BL35, BL37, BL39, BL41, BL50, BL56, BL57, BL222A, BL409T and BL512T, were immobilized covalently on wells of carboxylate-modified polystyrene microtiter plates as follows. Twenty-five μ l of the DNA probes described above which were

dissolved with sterile distilled water, was added to each of 15 wells which were used for a sample, in order shown in Figure 2. Next, 75 μ l of 0.2M EDC was added to each well and mixed. After the plates were sealed and incubated for 16 hours at room temperature, they were washed four times with PBS buffer 5 solution (7.5mM di-potassium hydrogenphosphate, 2.5mM potassium dihydrogenphosphate, 0.15M sodium chloride). Two hundreds μ l of 0.4N NaOH were added to each well and the plates were incubated for 1 hour at 37°C. The plates were washed four times with PBS buffer solution.

One hundred μ l of GMC buffer (0.25M di-sodium hydrogenphosphate, 7% SDS, 10 1% BSA, 0.5M EDTA, 0.03M phosphoric acid, 20% formamide) was added to each well of the microtiter plates and the plates were incubated for 5 min at 37°C. After incubation, the buffer was removed from each well. During incubation, 60 μ l of the amplified products described above, were denatured with an equivalent volume of 0.4 NaOH for 5 min at room temperature. After 15 denaturation, 1500 μ l of hybridization buffer was added to the denatured product, mixed and 100 μ l of them was added to each well. The microtiter plates were sealed and incubated for 1 hour at 37°C.

After the solution was removed from wells, the plates were washed five times with 2 \times SSC washing solution (0.3M sodium chloride, 0.03M tri-sodium 20 citrate), 100 μ l of peroxidase-conjugated streptavidin (Vector Laboratories) solution, diluted to 1/2000 in TTBS enzyme diluting solution (0.2M Tris-HCl(pH7.6), 0.5M sodium chloride, 0.5% Tween 20), was added to each well. The microtiter plates were sealed and incubated for 15 min at 37°C. After the solution was removed from wells, the plates were washed five 25 times with the washing solution described above, chromogenic substrate solution (3,3',5,5'-tetramethylbenzidine (TMB) solution:Kirkegaard & Perry Laboratories) was added and incubated for 30 min at 37°C. After incubation,

color development was stopped by adding 100 μ l of 1% SDS to each well and the absorbance was measured at 650 nm. The absorbance to each sequence is shown in Table 2. The absorbance for positive and negative signals was 1.0 and over, and under 0.5, respectively. By using these results, HLA-B40 5 allele typing for each sample (5 - 8) was performed according to the Typing Table shown in Figure 2. The typing results are shown in the bottom column of Table 2 as follows.

Table 2

Results of HLA-B40 allele typing (the absorbance at 650nm)

Well	SS0 probe	Sample 5	Sample 6	Sample 7	Sample 8
1	BL222A	1.846	1.671	1.742	1.849
2	BL34	2.126	2.148	2.182	2.239
3	BL35	0.088	0.082	0.083	0.093
4	BL4	1.966	1.870	1.800	1.976
5	BL5	0.154	0.161	0.142	0.205
6	BL24	1.711	1.744	1.671	2.018
7	BL25	0.050	0.051	0.056	0.067
8	BL512T	2.356	0.209	0.238	0.058
9	BL37	0.130	2.533	2.517	0.014
1 0	BL39	0.069	0.099	0.111	0.027
1 1	BL41	0.042	0.064	0.070	2.315
1 2	BL50	0.101	0.014	0.039	0.044
1 3	BL56	2.487	2.464	0.373	2.342
1 4	BL57	0.193	0.156	2.124	0.093
1 5	BL409T	0.038	0.050	0.287	0.031
HLA-B40 Allele type		B*4001	B*4002	B*4003	B*4006

Example 3 HLA-A antigen and allele typing

Leukocytes (Samples 9-12) which were isolated from peripheral blood (about 10ml) of normal subjects according to usual methods, were lysed in 5 500 μ l of guanidine thiocyanate buffer (4M guanidine thiocyanate, 25mM sodium citrate(pH7.0), 0.5% sodium N-lauroylsarcosinate, 1% mercaptoethanol). The solution was extracted twice with phenol to eliminate proteins. After mixing with 3 M sodium acetate buffer (pH5.2), genome DNAs were obtained by adding twice volume of chilled ethanol. By using this DNAs, typing of the 10 HLA-A antigens and alleles was performed as follows.

By using CGA011, CGA012 and 5'-biotinylated AIn3-66C for a primer pair, amplification of the region containing the exon 2, the intron 2 and the exon 15 3 of the HLA-A alleles from DNAs described above was performed by the PCR method. The reaction solution was composed of genomic DNAs (100 ng), 1.4 units of thermostable DNA polymerase which was pretreated with Taq Start™Antibody for 5 min at room temperature, 33.5mM Tris-HCl (pH 8.8), 8.8 mM ammonium sulfate, 1.5 mM magnesium chloride, 0.005% Tween 20, 200 μ M dNTPs, and each 1.7 μ M of a primer pair (the ratio of CGA011 to CGA012 is 4 to 1) in a final volume of 100 μ l. DNA amplification was performed by using 20 GeneAmp PCR system 9600 (Perkin Elmer) by initial denaturation at 95°C for 2 min followed by 5 cycles of denaturation for 25 s, annealing at 70°C for 45 s, extension at 72°C for 45 s followed by 36 cycles of denaturation for 25 s, annealing at 65°C for 50 s, extension at 72°C for 45 s.

5'-amino-modified DNA probes, A34, A239A, A238A, A257TC, A259AC, A282C, 25 A282CT, A290TR, A299T, A302GR, A355G, A414A, A448C, A468T, A489A, A502C, A526T, A538CG, A538TG, A539A, A539T, A555T, A570CG and A570GT, were immobilized covalently on wells of carboxylate-modified polystyrene

microtiter plates as follows. Twenty-five μ l of the DNA probes described above which were dissolved with sterile distilled water, was added to each of 23 wells which were used for a sample, in order shown in Figure 3. Next, 75 μ l of 0.2M EDC solution was added to each well and mixed. After the plates 5 were sealed and incubated for 16 hours at room temperature, they were washed four times with PBS buffer solution (7.5mM di-potassium hydrogenphosphate, 2.5mM potassium dihydrogenphosphate, 0.15M sodium chloride). Two hundreds μ l of 0.4N NaOH were added to each well and the plates were incubated for 1 hour at 37°C. The plates were washed four times with PBS buffer solution.

10 One hundred μ l of GMC buffer (0.25M di-sodium hydrogenphosphate, 7% SDS, 1% BSA, 0.5M EDTA, 0.03M phosphoric acid, 20% formamide) was added to each well of the microtiter plates and the plates were incubated at 37°C for 5 min. After incubation, the buffer of each well was removed from each well. During incubation, 96 μ l of the amplified products described above, were 15 denatured with an equivalent volume of 0.4 NaOH for 5 min at room temperature. After denaturation, 2400 μ l of hybridization buffer was added to the denatured products, mixed and 100 μ l of them was added to each well. The microtiter plates were sealed and incubated for 1 hour at 37°C.

After the solution was removed from wells, the plates were washed five 20 times with 2 \times SSC washing solution (0.3M sodium chloride, 0.03M tri-sodium citrate), 100 μ l of peroxidase-conjugated streptavidin (Boehringer Mannheim) solution, diluted to 1/2000 in TTBS enzyme diluting solution(0.2M Tris-HCl(pH7.6), 0.5M sodium chloride, 0.5% Tween 20), was added to each well. The microtiter plates were sealed and incubated for 15 min at 37°C. After 25 the solution was removed from wells, the plates were washed five times with the washing solution described above, chromogenic substrate solution (TMB solution : Kirkegaard & Perry Laboratories) was added and incubated for 30

min at 37°C. After incubation, color development was stopped by adding 100 μ l of 1% SDS to each well and the absorbance was measured at 650 nm. The absorbance for positive and negative signals was 1.0 and over, and under 0.5, respectively. By using these results, HLA-A antigen and allele typing for 5 each sample (9 - 12) was performed according to the Typing Table shown in Figure 3. The typing results are shown in the bottom column of Table 3 as follows.

Table 3

Results of HLA-A antigen and allele typing (the absorbance at 650nm)

Well	SSO probe	Sample 9	Sample 10	Sample 11	Sample 12
1	A468T	2.963	3.046	2.603	2.719
2	A570CG	0.087	2.951	0.081	2.847
3	A570GT	2.815	0.065	2.690	2.763
4	A282C+A282CT	1.950	2.825	2.538	2.552
5	A299T	0.111	0.119	0.279	0.162
6	A290TR	0.012	0.135	2.245	0.095
7	A355G	2.382	0.033	0.037	0.128
8	A259AC	0.048	0.063	0.095	2.127
9	A257TC	0.034	0.021	0.054	0.060
1 0	A238A	-0.016	0.011	1.907	0.041
1 1	A239A	0.037	0.052	0.061	0.187
1 2	A538CG	0.012	0.025	0.017	0.065
1 3	A555T	0.068	0.038	0.066	0.090
1 4	A539T	2.480	0.048	1.618	0.093
1 5	A539A	0.111	2.513	0.205	2.402
1 6	A526T	0.023	0.046	0.105	0.065
1 7	A538TG	0.109	0.118	0.092	2.125
1 8	A302GR	-0.020	0.169	0.030	0.237
1 9	A34	2.186	0.121	1.441	2.271
2 0	A414A	0.031	0.127	0.079	0.095
2 1	A448C	0.232	0.091	0.073	2.412
2 2	A489A	2.896	0.100	0.051	0.276
2 3	A502C	0.017	0.135	1.401	2.517
HLA-A antigen and Allele type		A2/-	A24/-	A*31012/-	A24/26

Example 4 HLA-B antigen and allele typing

Leukocytes (Samples 13-16) which were isolated from peripheral blood (about 10ml) of normal subjects according to usual methods, were lysed in 500 μ l of guanidine thiocyanate buffer (4M guanidine thiocyanate, 25mM sodium citrate(pH7.0), 0.5% sodium N-lauroylsarcosinate, 1% mercaptoethanol). The solution was extracted twice with phenol to eliminate proteins. After mixing with 3 M sodium acetate buffer (pH5.2), genome DNAs were obtained by adding twice volume of chilled ethanol. By using the DNAs, typing of the HLA-B antigen and allele was performed as follows.

10 By using 5BIN1-TA, 5BIN1-CG and 5'-biotinylated 3BIN3-37 for a primer pair, amplification of the region containing the exon 2, the intron 2 and the exon 3 of the HLA-B alleles from DNAs described above was performed by the PCR method. The reaction solution was composed of genomic DNAs (100 ng), 1.4 units of thermostable DNA polymerase which was pretreated with Taq 15 StartTMAntibody for 5 min at room temperature, 67mM Tris-HCl (pH 8.8), 16.6 mM ammonium sulfate, 1.5 mM magnesium chloride, 0.01% Tween 20, 10% DMSO, 200 μ M dNTPs, and each 1.7 μ M of a primer pair (the ratio of 5BIN1-TA to 5BIN1-CG is 2 to 3) in a final volume of 100 μ l. DNA amplification was performed by using GeneAmp PCR system 9600 (Perkin Elmer) by initial 20 denaturation at 95°C for 2 min followed by 5 cycles of denaturation for 25 s, annealing at 70°C for 45 s, extension at 72°C for 45 s followed by 36 cycles of denaturation for 25 s, annealing at 65°C for 50 s, extension at 72°C for 45 s.

5'-amino-modified DNA probes, BL1, BL3, BL4, BL9, BL10, BL11, BL34, BL36, 25 BL37, BL38, BL39R, BL40, BL41, BL42, BL77, BL78, BL79, BL226G, BL263T, BL272A, BL527A, BL538CG, BL538G and BL570GT, were immobilized covalently on wells of carboxylate-modified polystyrene microtiter plates as follows.

Twenty-five μ l of the DNA probes described above which were dissolved with sterile distilled water, was added to each of 23 wells which were used for a sample, in order shown in Figures 4 and 5. Next, 75 μ l of 0.2M EDC was added to each well and mixed. After the plates were sealed and incubated 5 for 16 hours, they were washed four times with PBS buffer solution (7.5mM di-potassium hydrogenphosphate, 2.5mM potassium dihydrogenphosphate, 0.15M sodium chloride). Two hundreds μ l of 0.4N NaOH were added to each well and the plates were incubated for 1 hour at 37°C. The plates were washed four times with PBS buffer solution.

10 One hundred μ l of GMC buffer (0.25M di-sodium hydrogenphosphate, 7% SDS, 1% BSA, 0.5M EDTA, 0.03M phosphoric acid, 20% formamide) was added to each well of the microtiter plates and the plates were incubated for 5 min at 37°C. After incubation, the buffer was removed from each well. During incubation, 96 μ l of the amplified products described above, were denatured with an 15 equivalent volume of 0.4 NaOH for 5 min at room temperature. After denaturation, 2400 μ l of hybridization buffer was added to the denatured products, mixed and 100 μ l of them was added to each well. The microtiter plates were sealed and incubated for 1 hour at 37°C.

After the solution was removed from wells, the plates were washed five 20 times with 2 \times SSC washing solution (0.3M sodium chloride, 0.03M tri-sodium citrate), 100 μ l of peroxidase-conjugated streptavidin (Boehringer Mannheim) solution, diluted to 1/2000 in TTBS enzyme diluting solution(0.2M Tris-HCl(pH7.6), 0.5M sodium chloride, 0.5% Tween 20), was added to each well. The microtiter plates were sealed and incubated for 15 min at 37°C. After 25 the solution was removed from wells, the plates were washed five times with the washing solution described above, chromogenic substrate solution (TMB solution : Kirkegaard & Perry Laboratories) was added and incubated for 30

min at 37°C. After incubation, color development was stopped by adding 100 μ l of 1% SDS to each well and the absorbance was measured at 650 nm. The absorbance for positive and negative signals was 1.0 and over, and under 0.5, respectively. By using these results, HLA-B antigen and allele typing for 5 each sample (13 - 16) was performed according to the Typing Tables shown in Figures 4 and 5. The typing results are shown in the bottom column of Table 4 as follows.

Table 4

Results of HLA-B antigen and allele typing (the absorbance at 650nm)

Well	SSO probe	Sample 12	Sample 14	Sample 15	Sample 16
1	BL36	0.064	0.131	0.101	0.087
2	BL37	2.155	0.055	0.021	0.009
3	BL38	0.447	0.150	0.110	0.071
4	BL39R	0.147	1.476	0.143	0.103
5	BL40	0.026	0.040	0.290	0.211
6	BL41	0.064	0.062	2.650	2.213
7	BL42	0.268	0.235	0.237	0.120
8	BL77	2.564	0.038	0.075	0.128
9	BL78	0.104	2.559	2.549	2.627
1 0	BL79	0.115	0.232	0.199	2.316
1 1	BL1	0.080	1.065	0.176	0.241
1 2	BL9	1.787	0.124	0.058	1.142
1 3	BL3	0.173	0.163	0.141	0.144
1 4	BL4	0.055	1.720	0.142	0.215
1 5	BL10	2.256	0.051	0.066	1.847
1 6	BL11	0.178	0.064	0.264	0.054
1 7	BL272A	0.038	0.105	0.044	0.071
1 8	BL226G	0.034	0.163	0.137	0.102
1 9	BL263TA	0.005	0.173	0.048	0.012
2 0	BL34	1.992	0.168	0.186	2.446
2 1	BL527A	2.674	0.383	2.369	1.948
2 2	BL538CG+BL538G	2.619	0.311	0.354	0.356
2 3	BL570GT	2.538	0.421	2.645	2.821

HLA-B antigen and allele type	B7/-	B*4403/-	B51/-	B51/55
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Example 5 HLA-C allele typing

Leukocytes (Samples 17-20) which were isolated from peripheral blood (about 10ml) of normal subjects according to usual methods, were lysed in 500 μ l of guanidine thiocyanate buffer (4M guanidine thiocyanate, 25mM sodium citrate(pH7.0), 0.5% sodium N-lauroylsarcosinate, 1% mercaptoethanol).
5 The solution was extracted twice with phenol to eliminate proteins. After mixing with 3M sodium acetate buffer (pH5.2), genome DNAs were obtained by adding twice volume of chilled ethanol. By using the DNAs, typing of the HLA-C alleles was performed as follows.

10 By using 5BCIn37-24C, 5BCIn-37-24g and 5'-biotinylated 5BCIn37-34g2 for a primer pair, amplification of the region containing the exon 2, the intron 2 and the exon 3 of the HLA-C alleles from DNAs described above was performed by the PCR method. The reaction solution was composed of genomic DNAs (100 ng), 1.4 units of thermostable DNA polymerase which was pretreated with Taq
15 StartTMAntibody for 5 min at room temperature, 33.5mM Tris-HCl (pH 8.8), 8.8 mM ammonium sulfate, 1.5 mM magnesium chloride, 0.005% Tween 20, 200 μ M dNTPs, and each 1.7 μ M of a primer pair in a final volume of 100 μ l. DNA amplification was performed by using GeneAmp PCR system 9600 (Perkin Elmer) by initial denaturation at 95°C for 2 min followed by 5 cycles of denaturation
20 for 25 s, annealing at 70°C for 45 s, extension at 72°C for 45 s followed by 36 cycles of denaturation for 25 s, annealing at 65°C for 50 s, extension at 72°C for 45 s.

5'-amino-modified DNA probes, 201g1, C206gR, A-12, RA-2, A-3, RA-41, A-54, B-1, RB-28, C-12, C-24, C-33, C-43, 134-g, 134-A2, 353TCA1, R341A, 343A,
25 R343g3, 353TCC, 361T1, 361T368g, 361T368T1, 369C, 387g1, 526AC2 and 538gAC, were immobilized covalently on wells of carboxylate-modified polystyrene microtiter plates as follows. Twenty-five μ l of the DNA probes described

above which were dissolved with sterile distilled water, was added to each of 23 wells which were used for a sample, in order shown in Figure 6. Next, 75 μ l of 0.2M EDC solution was added to each well, mixed and sealed. After the plates were sealed and incubated for 16 hours, they were washed four times 5 with PBS buffer solution (7.5mM di-potassium hydrogenphosphate, 2.5mM potassium dihydrogenphosphate, 0.15M sodium chloride). Two hundreds μ l of 0.4N NaOH were added to each well and the plates were incubated for 1 hour at 37°C. The plates were washed four times with PBS buffer solution.

One hundred μ l of GMC buffer (0.25M di-sodium hydrogenphosphate, 7% SDS, 10 1% BSA, 0.5M EDTA, 0.03M phosphoric acid, 20% formamide) was added to each well of the microtiter plates and the plates were incubated for 5 min at 37°C. After incubation, the buffer was removed from each well. During incubation, 96 μ l of the amplified products described above, were denatured with an equivalent volume of 0.4 NaOH for 5 min at room temperature. After 15 denaturation, 2400 μ l of hybridization buffer solution was added to the denatured products, mixed and 100 μ l of them was added to each well. The microtiter plates were sealed and incubated for 1 hour at 37°C.

After the solution was removed from wells, the plates were washed five times with 2 \times SSC washing solution(0.3M sodium chloride, 0.03M tri-sodium 20 citrate). One hundred μ l of peroxidase-conjugated streptavidin (Boehringer Mannheim) solution, diluted to 1/2000 in TTBS enzyme diluting solution(0.2M Tris-HCl(pH7.6), 0.5M sodium chloride, 0.5% Tween 20), was added to each well. The microtiter plates were sealed and incubated for 15 min at 37°C. After the solution was removed from wells, the plates were washed five times with 25 the washing solution described above, chromogenic substrate solution (TMB solution : Kirkegaard & Perry Laboratories) was added and incubated for 30 min at 37°C. After incubation, color development was stopped by adding 100

μ l of 1% SDS to each well and the absorbance was measured at 650 mm. The absorbance for positive and negative signals was 1.0 and over, and under 0.5, respectively. By using these results, HLA-C allele typing for each sample (17 - 20) was performed according to the Typing Table shown in Figure 6. The 5 typing results are shown in the bottom column of Table 5 as follows.

Table 5

Results of HLA-C allele typing (the absorbance at 650nm)

Well	SSO probe	Sample 17	Sample 18	Sample 19	Sample 20
1	C206gR	2.080	2.069	2.003	1.871
2	A-12	2.165	-0.024	-0.029	1.805
3	RA-2	0.020	1.992	0.120	1.979
4	A-3	0.069	0.038	0.052	0.081
5	RA-41	0.008	0.033	0.121	0.102
6	A-54	-0.012	0.194	2.080	0.059
7	B-1	0.202	0.124	0.145	0.233
8	RB-28	2.403	1.640	1.716	1.998
9	C-12	1.855	0.045	0.019	1.739
1 0	C-24	0.138	0.064	2.002	0.287
1 1	C-33	0.086	2.563	0.077	2.181
1 2	C-43	0.113	0.182	0.137	0.174
1 3	134-g	1.594	0.089	1.763	1.384
1 4	134-A2	0.049	2.096	0.291	1.380
1 5	343A	0.021	2.672	0.047	1.480
1 6	R343g3+R341A	2.562	0.292	2.717	1.928
1 7	353TCA1	0.001	2.551	0.157	1.740
1 8	353TCC	0.021	-0.002	0.092	0.006
1 9	201g1	1.209	1.679	0.176	1.225
2 0	369C	0.055	0.183	2.640	0.163
2 1	361T1+361T368g +361T368T1	2.345	0.040	0.048	1.885
2 2	387g1	0.028	0.054	0.015	0.019
2 3	526AC2+538gAC	0.090	0.074	0.124	0.092

HLA-C allele type	C*0102/-	C*0303/-	C*1202/-	C*0102/ 0303
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Industrial Applicability

By this invention, a single HLA class I antigen or allele is determined by combining PCR amplification using a primer pair which can amplify all the HLA-A alleles, all the HLA-B alleles or all the HLA-C alleles or which is specific to the common sequence to alleles of the specific group consisting of the specific HLA-A alleles or the specific HLA-B alleles, with reverse hybridization analysis using DNA probes to enable to specifically hybridize with the sequence of at least a specific HLA-A allele, at least a specific HLA-B allele or at least a specific HLA-C allele, which are covalently immobilized on wells of microtiter plates. Therefore, it can solve problems from the viewpoint of manipulation of HLA class I loci antigen typing by the classical serological method, and can classify at the allele level (allele typing) the class I antigens or subtypes to be unable to distinguish and classify by the classical method. Furthermore, at the same time, it can solve problems from the viewpoint of manipulation and resolution of HLA class I allele typing. Namely, this invention enables us to easily mechanize and automate detection and determination of the HLA class I alleles. This invention offers a method, a reagent and a kit for typing of the HLA class I alleles, which are useful for judgement of compatibility between a donor and a recipient in organ transplantation and for association analysis between the HLA class I genes and various kinds of diseases in the clinical and medical field.